COMPOSITION OF EXOPOLYMER PARTICLES PRODUCED BY MARINE DIATOMS

An Undergraduate Research Scholars Thesis

By

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ABSTRACT

Composition of Exopolymer Particles Produced By Marine Diatoms.

(May 2013)

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Microbial processes in the sea are an essential component of the Earth's biosphere. Planktonic microorganisms constitute the majority of photosynthetic primary producers in the ocean in terms of both abundance and biomass. Planktonic photosynthetic microorganisms (phytoplankton) fix approximately half of the organic carbon on Earth and therefore an understanding of the fate of that carbon is essential to understand the functioning of the Earth. Impacts of finding great amounts of DNA not enclosed in a biotic organism could heavily impact species differentiation in the ocean. Exopolymer particles are excreted by phytoplankton, including diatoms.

These particles are understood to contain carbohydrates and protein that can potentially serve as a food source to bacteria in the surface of the water column, also impacting the marine organic carbon cycle. This study explored the composition of the exopolymer particles produced by marine diatoms with the aid of dyes, fluorescent stains, and in the future, lectins. My central hypothesis was that marine exopolymer particles have a complex heterogeneous polymeric structure containing polysaccharides, proteins and extracellular nucleic acids. Preliminary results show that DNA is part of diatom's exopolymer carbohydrate particles and the hypothesis was proven correct, but the results from the experiment were affected by many other factors. This

decreases the confidence for the results. Results also show that protein particles do contain acid polysaccharides but that the polysaccharides that make up TEP do not contain lots of glucose and mannose, derived from staining using concanavalin A-FITC. There is reason to believe that the acidity of the Alcian Blue affected the results of the abundance of glucose and mannose in the TEP samples however.

NOMENCLATURE

TEP: transparent exopolymer particles

CSP: Coomassie Staining Particles

eDNA: extracellular DNA

AB: Alcian Blue stain

CBB: Coomassie Brilliant Blue stain

CCA: concanavalin A-FITC

T.W.: *Thalassiosira weissflogii*

O.A.: *Odontella aurita*

S.C.: *Skeletonema costatum*

C.W.: *Coscinodiscus wailesii*

CHAPTER I

INTRODUCTION

Phytoplankton play a huge role in the eutrophic zone (generally < 100 m deep because of the transmission of light and the ability to support life) of the world's oceans. Diatoms are siliceous phytoplankton that reside throughout the oceans, typically in colder waters (above 50 degrees latitude) and also in regions of upwelling. Diatoms are responsible for an approximate 20 Pg $year^{-1}$ (1 Pg = 10^{15} g) of the primary production in the ocean, which is between 40-45% of the total primary production (Thornton, 2002). In comparison, the net productivity is estimated to be 45 to 55 Pg $year^{-1}$ of carbon in the global ocean (Longhurst et al. 1995). Within the oceans, 1 to 2 Pg of carbon per year is determined to be from living organisms, from whales to cyanobacteria (Falkowski et al. 2000). This may seem like an enormous quantity, but it has been estimated that the amount of carbon in the form of exopolymer particles in the ocean is 70 Pg, which is greater than the net production produced by all the living organisms in the ocean (Verdugo et al. 2004). The oceans hold much of the world's carbon (662 Pg of just dissolved organic carbon), and it is crucial that we learn how the ocean releases, absorbs, and stores carbon (Hansell et al. 2009). Microorganisms use photosynthesis for energy, and excrete polymers that clump together. The reason for the excretion of the polymers is unknown. These clumps form transparent amorphous shapes that are described as exopolymer particles. The particles released by diatoms are categorized by features such as solubility, molecular weight, and structure (Thornton, 2002). One of the two most studied exopolymer particles are known as transparent exopolymer particles (TEP), which are made from polysaccharides as they stain with Alcian blue dye (Alldredge et al. 1993). TEP is shown in Figure 1 below.

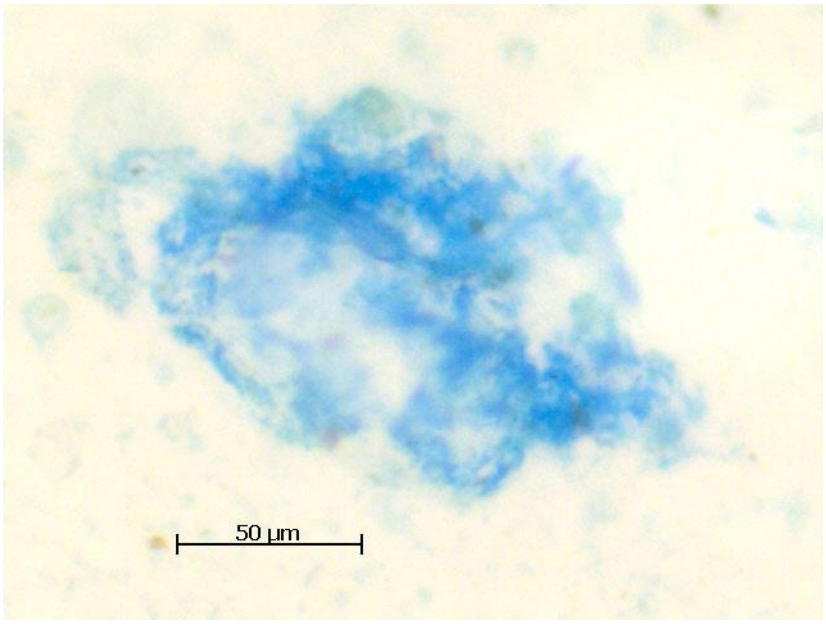


Figure 1: *Light micrograph of TEP from the Pacific Ocean stained with Alcian Blue*: Photograph taken by Daniel C. O. Thornton.

TEP is an important resource for bacteria in areas of low nutrient concentrations (Arnous, 2010), since diatoms produce more TEP with lower amounts of nutrients. The other important class of expolymer particles are Coomassie staining particles (CSP), which stain with Coomassie Brilliant Blue dye, indicating that they contain proteins (Long and Azam, 1996). Exopolymer particles, such as TEP, make a significant contribution in the carbon cycle as they remove organic carbon from the surface ocean into the deep ocean as they sink.

The composition of exopolymer particles at this moment is accepted to be homogeneous. There have been no studies in which particles have been stained with both Alcian blue (testing for carbohydrates) and Coomassie Brilliant Blue (testing for proteins) as both dyes stain a similar shade of blue. Therefore, it is not known whether TEP and CSP are distinct classes of exopolymer particle or whether they are the same particles. My objective is to characterize CSP

and TEP using dyes, fluorescent stains, and lectins conjugated to fluorophors to stain specific components of the exopolymer particles. My central hypothesis is that exopolymer particles are heterogeneous particles containing a mixture of DNA, acid polysaccharides, and proteins.

CHAPTER II

METHODS

Hypotheses

Exopolymer Particles contain extracellular DNA (eDNA).

The composition of exopolymer particles has not been extensively studied in marine ecosystems. Preliminary observations (Chen and Thornton, pers.com) from the Earth System and Microbial Ecology Laboratory have indicated the presence of eDNA in TEP. By employing specific fluorescent nucleic acid stains, I will determine whether TEP contain eDNA. A huge factor is that DNA in exopolymer particles can give false positives to the presence of organisms in the ecosystem. In 2004, Venter et al wrote a paper on all the organisms in the Sargasso Sea by obtaining their genomes from samples (Venter et al 2004). Based on this study, if there was eDNA in the water column, a genome could be notified without the organism being there or being alive in the water column.

TEP contain monosaccharides commonly found dissolved in the ocean.

While Alcian blue staining shows that TEP contain polysaccharides, there is little information on the monosaccharides that contribute to these polymers or their distribution within individual exopolymer particles. Lectins will be used to determine the individual monosaccharides making up TEP. I will choose which lectins to use in this work based on the known carbohydrate composition of diatoms (Aluwihare and Repeta, 1999).

TEP and CSP are chemically heterogeneous exopolymer particles.

All previous studies have assumed that TEP and CSP are strictly homogenous particles (Passow, 2002). Preliminary observations (Chen and Thornton, personal communication) indicate that exopolymer particles are heterogeneous with a complex structure. I will use a combination of stains and lectins to determine if the composition is heterogeneous.

Procedure

The abundance of TEP (transparent exopolymer particles) was determined using three cultures of the diatom species *Odontella aurita.* The cultures were grown in a total two liters of Harrison's sea water medium (Berges et al 2001). There were four cultures created, each containing 300mL of autoclaved seawater. Into each culture, 1 mL of *Odontella aurita* from a previously grown healthy culture was inserted. There were four created as a backup in case one culture died or failed, but only three will be used in the experiment.

To begin the testing of the hypotheses, growth in the culture was measured by cell counts (Anderson 2005). Cell counts were taken every few days during the growth process, even if samples were not taken for staining. For counts, a 1 mL sample of each culture was taken and mixed with one drop of Lugol's iodine (Anderson 2005). This is simply just to kill the cells and preserve them in dark gold color to make it easier to see for the counts. Individual samples were mounted on a thick haemocytometer slide and counted using a transmitted light microscope (Anderson 2005).

During a main sampling day, samples to test for the composition of TEP during the different stages of growth were also taken. The stains for the components of TEP being tested were Alcian

Blue (for acidic polysaccharides), DAPI (for DNA but cell membrane permeable), and SYTOX Green (for DNA but not cell membrane permeable). To check for artifacts in the fluorescent staining, Xanthan gum (which is a simple carbohydrate solution and provided a control) was tested along with Alcian Blue, DAPI, and SYTOX Green, to determine the validity of the fluorescence. DNA stains need dark and cold environments to function at full capacity, so storage was in the freezer in the lab (Gossart 1999). To stain a 1 mL sample with SYTOX Green (.01 dilution), 1 mL of culture was mixed with .4 mL of SYTOX Green, and then allowed to bind in the refrigerator for at least 20 minutes, typically closer to 30 minutes but up to an hour at most. DAPI staining was similar, but for a 1 mL sample of culture, .1 mL of DAPI was mixed in and allowed to bind (Gossart 1999). The bind time was consistent between SYTOX Green samples and DAPI samples. All sampling was completed under a sterile hood (Gossart 1999). Using SYTOX Green, a fluorescent green nucleic acid stain, eDNA will be visualized in the sample. Using DAPI in comparison to SYOTX Green, eDNA will be able to be confirmed as eDNA and not mistaken for DNA present inside associated bacteria and diatoms in the sample.

During each sampling day, there were a total of 18 slides created, and within each individual culture, there were six slides:

1) culture only
2) Alcian Blue only
3) SYTOX Green only
4) DAPI only
5) Alcian Blue and SYTOX Green
6) Alcian Blue and DAPI

Each sample was filtered on a 0.4 μm Nucleopore filter with NaCl solution and mounted on a Cyto-Clear slide (Alldredge et al 1993). There was an initial sampling day at the beginning of the experiment to complete checks. The two main sampling days were one month apart.

On the first day, samples were filtered after staining with the nucleic acid stains. Filtering consisted of 1.6 mL of NaCl (added first to filter) mixed with .4 mL of the sample. This amount was determined from the age of the culture and samples taken to see the amount of cells on the slides to get an accurate estimate of TEP and cells. Checks such as this one used samples taken from the fourth culture created at the beginning of the experiment. The nucleic acid stains were bound to the samples before filtering. Alcian Blue on the other hand, was added during the filtering process. Alcian Blue was filtered for precipitates the morning of the sampling. For the total filtered 2 mL of culture mixed with NaCl, 1 mL of Alcian Blue was filtered then rinsed out (Alldrege et al 1993). Finally, there were two rinses of the filter using UHP water before the pressure was released and the filter could be mounted on the Cyto-Clear Slide (Alldrege et al 1993).

To analyze the particles of TEP throughout the culture, results will be recorded as transmitted and fluorescent light micrographs taken with cameras attached to a fluorescence microscope (Axioplan 2, Zeiss Imaging). The images will be analyzed using ImageJ software (National Institutes of Health). The pictures used were from the Alcian Blue only slides from each culture on each sampling day, ending in a total of six slides to be analyzed quantitatively. To prevent bias, ten pictures were taken from every slide, starting in the middle of the filter on the left side. Each picture was adjacent to the picture before, moving towards the right side of the filter. This

was done for every slide. The settings were set to be the same in every picture taken: linear at 70%, color offset at 0.00 (denatures the original colors in the photo, making the colors brighter or softer), exposure at 60ms (the amount of light allowed though), and the halogen bulb set at 7 brightness (how bright the light is in the background). The pictures were then analyzed in Image J.

In Image J, the settings for each picture had to be determined. The scale was converted from pixels to μm (1 pixel = 0.642 μm). In Image J, the red channel was applied for TEP while the threshold was set as 'triangle' and around 200. The 'triangle' method uses the nearest three points to determine if the particle is actually part of the result or just a random particle. In the analysis, holes were not included and particles overlapping the edges of the frame were taken into account. Edges would just be excluded if taken the average size and such measurements. The area range for the particles was taken at 20μm-Infinity.

For the second portion of this experiment, TEP and CSP were analyzed for glucose and mannose. There were four species tested: (*Thalassiosira weissflogii, Odontella aurita, Skeletonema costatum, Coscinodiscus wailesii*). First, a sample is filtered and stained with Alcian blue dye to confirm the presence of TEP and Coomassie Brilliant Blue stain to test for CSP. After TEP are confirmed in the samples (Alldredge, 1993), a fluorescent lectin, concanavalin A-FITC, will be added. Lectins bind to specific monosaccharides (Wigglesworth-Cooksey and Cooksey 2004). Concanavalin A-FITC binds to glucose and mannose (Wigglesworth-Cooksey and Cooksey 2004). Six week old cultures were sampled and stained with Alcian Blue, Coomassie Brilliant Blue, and the lectin, concanavalin A-FITC. The lectin was allowed to bind to the sample for one

hour (Wigglesworth-Cooksey and Cooksey, 2004). Otherwise, the procedures were the same as the first experiments. There were three slides made from each species. Slides were created exactly the same as the first experiment as well. Each species had an Alcian Blue and concanavalin A-FITC, Coomassie Brilliant Blue and concanavalin A-FITC, and a concanavalin A-FITC only slide. Each slide was qualitatively analyzed with photographs just as in the first experiment.

CHAPTER III

RESULTS

Growth in the cultures was measured by cell counts at multiple dates over the course of this experiment. The cultures increased over time, as shown in Figure 2.

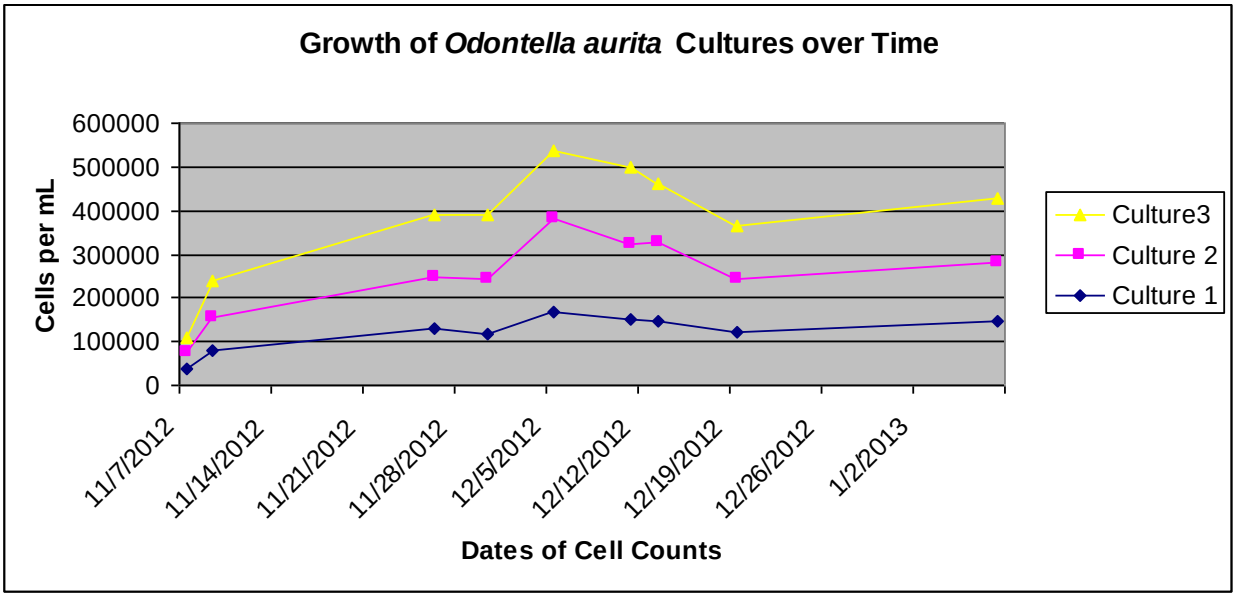


Figure 2: Growth of *Odontella aurita* Cultures over Time

TEP concentrations were quantified at two dates within the experiment, a month apart (Figure 3). Also, TEP concentration was quantified using the abundance of cells for the estimated amount of TEP produced by each cell in the separate cultures (Figure 4).

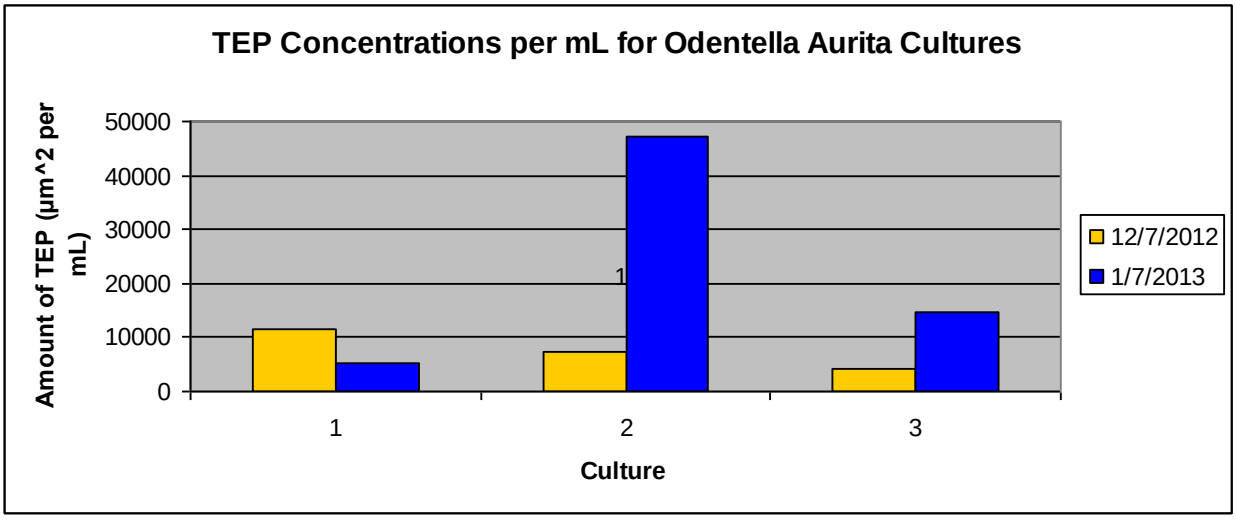


Figure 3: Total area of TEP for each Culture at the two Sampling Dates

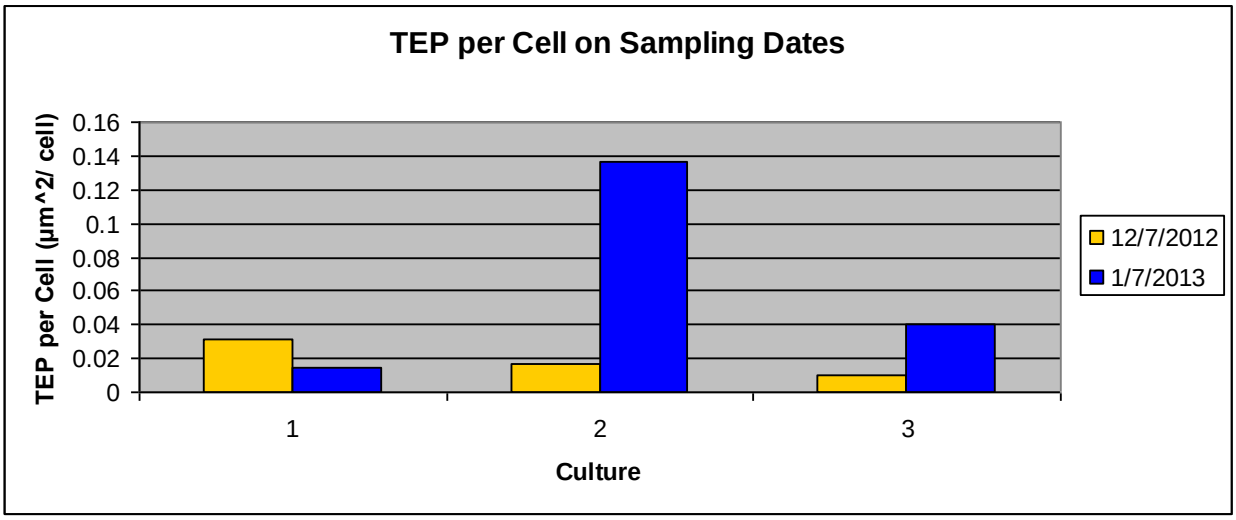


Figure 4: Total Area of TEP per individual cell in cultures based on cell counts

For evidence of heterogeneity of the TEP particles using nucleic acid stains, pictures were taken of the double stained particles, once under fluorescent lighting to show potential DNA staining from DAPI or SYTOX Green then once under halogen lighting to show Alcian Blue staining, as shown in Figures 5-10.

Figure 5

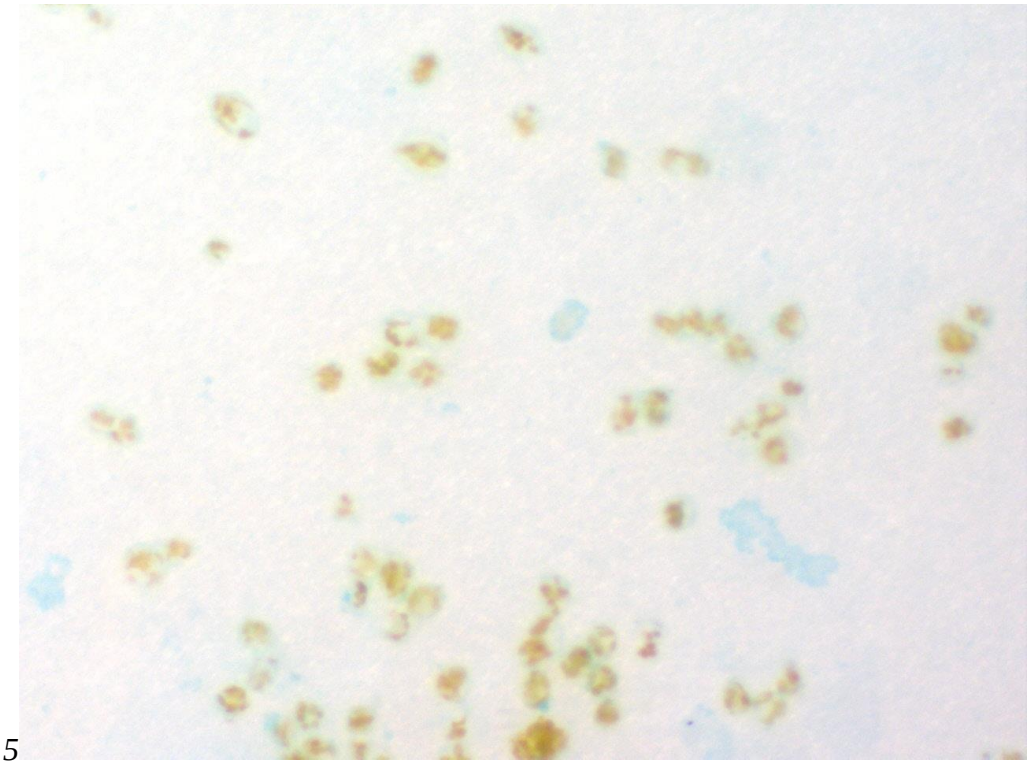
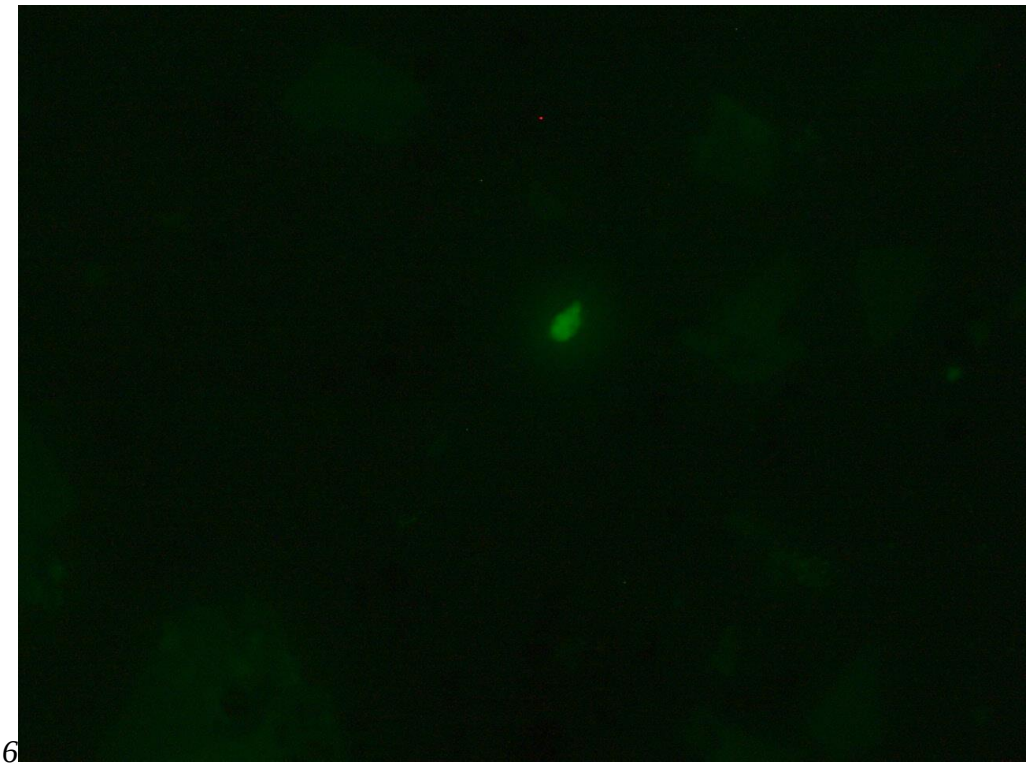


Figure 6



Culture 1: 12/07/12

Figure 5: *Single Fluorescing TEP under Halogen*: Halogen image of culture 1 and the TEP particles stained with Alcian Blue.

Figure 6: Single Fluorescing TEP with SYTOX Green: The same image under fluorescent light and stained with SYTOX Green to show only one TEP particle fluorescing under fluorescence. The lighter fluorescing particles are artifacts from the medium.

Figure 7

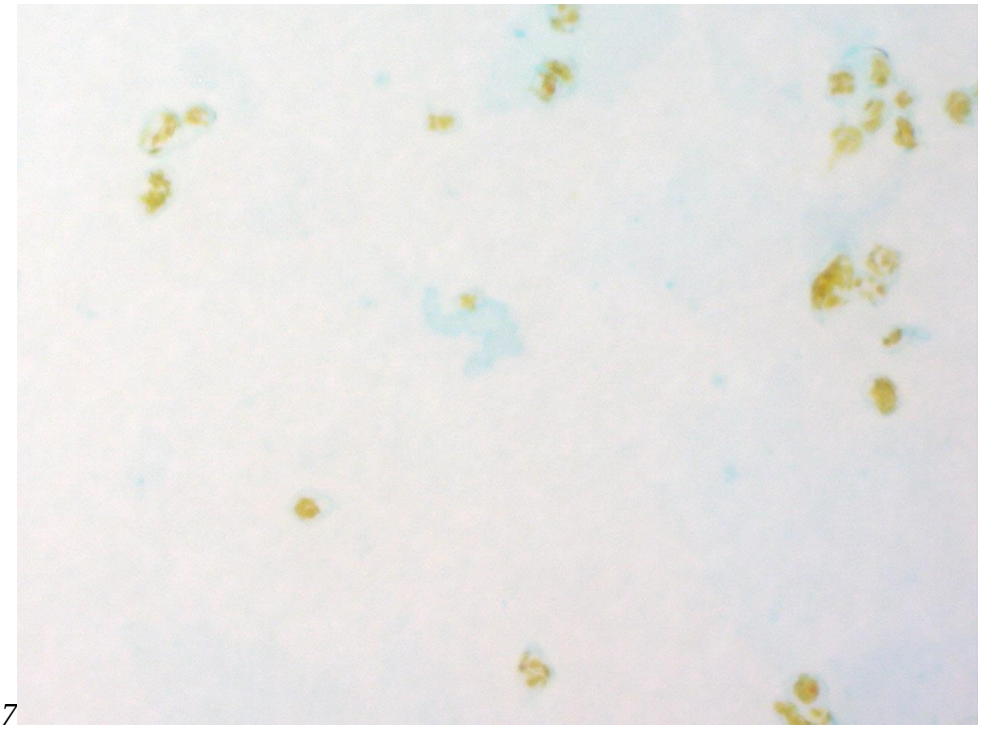
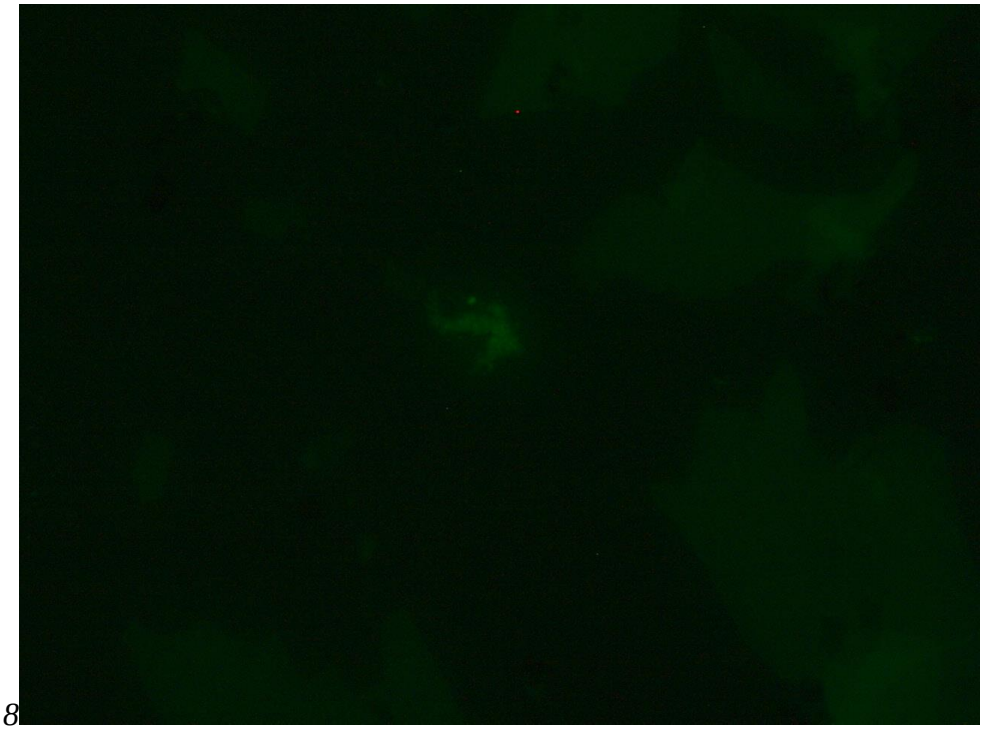


Figure 8



Culture 1: 12/07/12

Figure 7: *Fluorescing TEP under Halogen*: Halogen image of culture 1 and the TEP particles stained with Alcian Blue.

Figure 8: *Fluorescing TEP with SYTOX Green:* is the same image under fluorescent light and stained with SYTOX Green to show the same TEP particle fluorescing under fluorescence. The lighter fluorescing particles are artifacts from the medium.

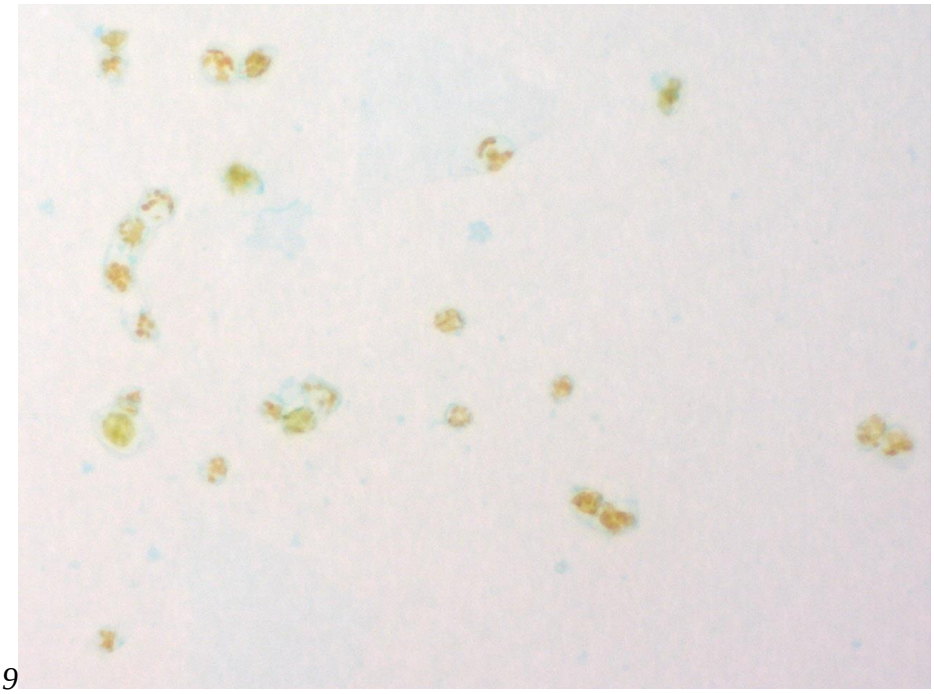


Figure 9

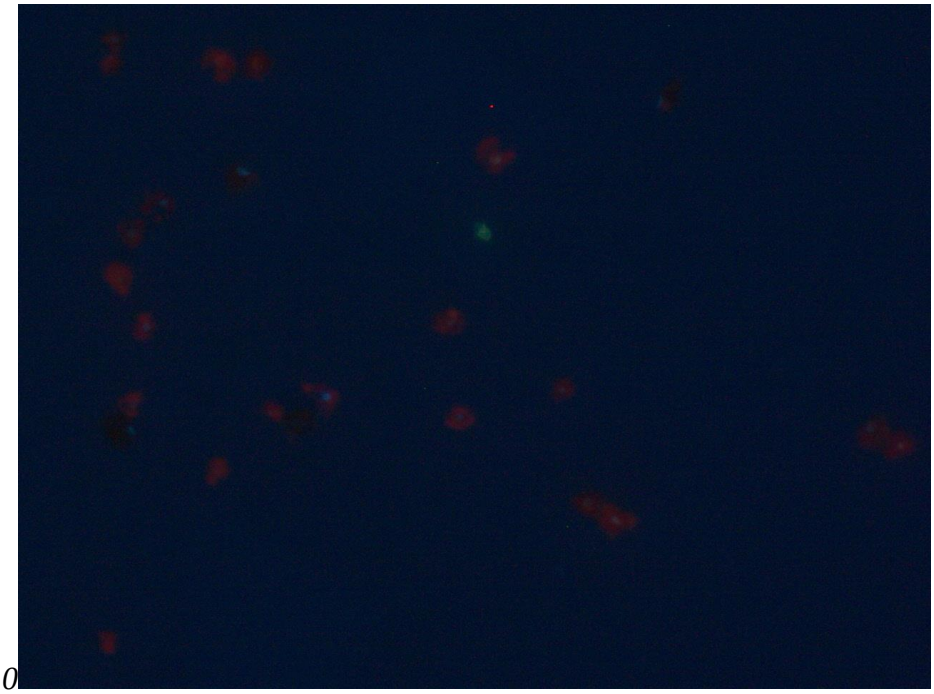


Figure 10 Culture 1: 12/07/12

Figure 9: *Single TEP containing DNA under Halogen*: Halogen image of culture 1 and the TEP particles stained with Alcian Blue.

Figure 10: *Single TEP containing DNA with DAPI*: Same image under fluorescent light and stained with DAPI to show only one TEP particle fluorescing under fluorescence.

In some of the issues discovered in the analysis, artifacts also fluoresced, as shown in Figures 11 and 12.

Figure 11

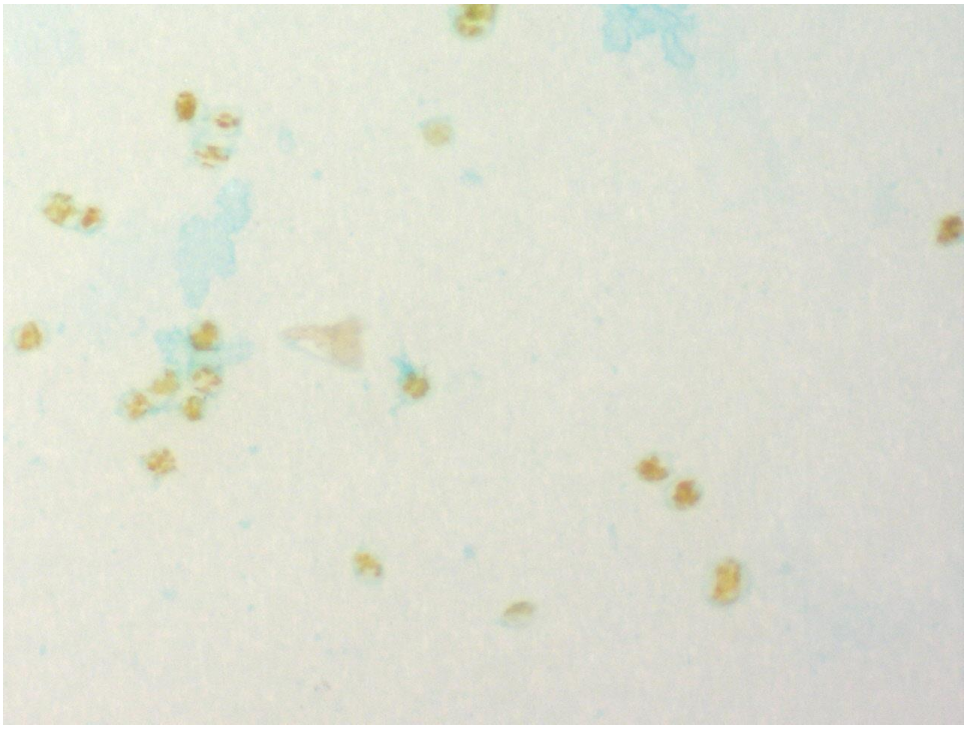
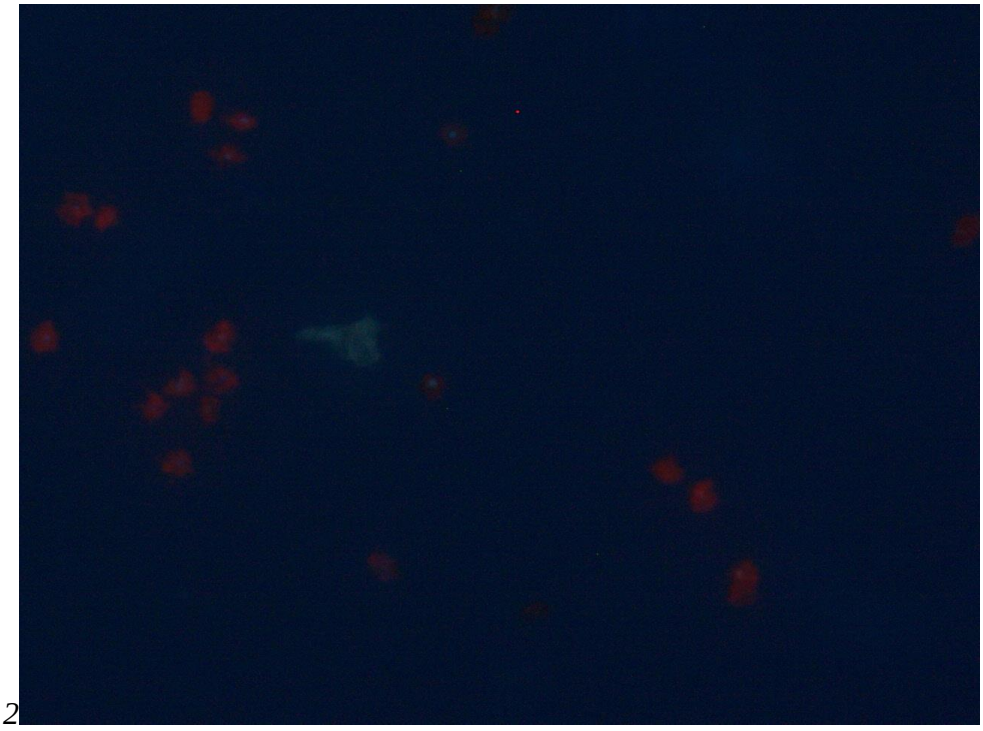


Figure 12



Culture 1: 12/07/12

Figure 11: *Artifact under Halogen*: Halogen image of culture 1 and the TEP particles stained with Alcian Blue.

Figure 12: *Artifact Fluorescing with DAPI*: Same image under fluorescent light and stained with DAPI to show no TEP particles fluorescing under fluorescence but an artifact that is fluorescing.

These pictures show that DNA is present within some of the TEP particles, but not in a large amount of them. Due to the inconsistencies within the growth experiment of the cultures, another experiment would need to be done to confirm the results.

The second portion of this study was determining if there were acid polysaccharides derived from mannose or glucose in the both TEP and CSP. There were four different species tested: (*Thalassiosira weissflogii, Odontella aurita, Skeletonema costatum, Coscinodiscus wailesii*).

Four Cultures and their Exopolymer Particles Abundance of Glucose and Mannose

Species and Staining	Abundant Fluorescence	Trace Fluorescence	No Fluorescence or Artifact Fluorescing
OA: Stained with AB and CCA	X		
OA: Stained with CBB and CCA	X		
OA: Stained with CCA	X		
TW: Stained with AB and CCA	X		
TW: Stained with CBB and CCA			X
TW: Stained with CCA	X		
SC: Stained with AB and CCA	X		
SC: Stained with CBB and CCA			X
SC: Stained with CCA		X	
CW: Stained with AB and CCA		X	
CW: Stained with CBB and CCA			X
CW: Stained with CCA	X		

Table 1: Four Cultures and their Exopolymer Particles Abundance of Glucose and Mannose: This table shows the results of the amount of fluorescence based on the type of organism and the

stains that were in the sample. Abundant fluorescing shows a significant amount of glucose and mannose in the sample. No fluorescing or artifact fluorescing is determined to contain little to no glucose and mannose in the sample.

In the results, there was evidence that there is glucose and mannose in the TEP particles, which was expected since TEP consist of clumps of acid polysaccharides, as shown in Figures 13-16.

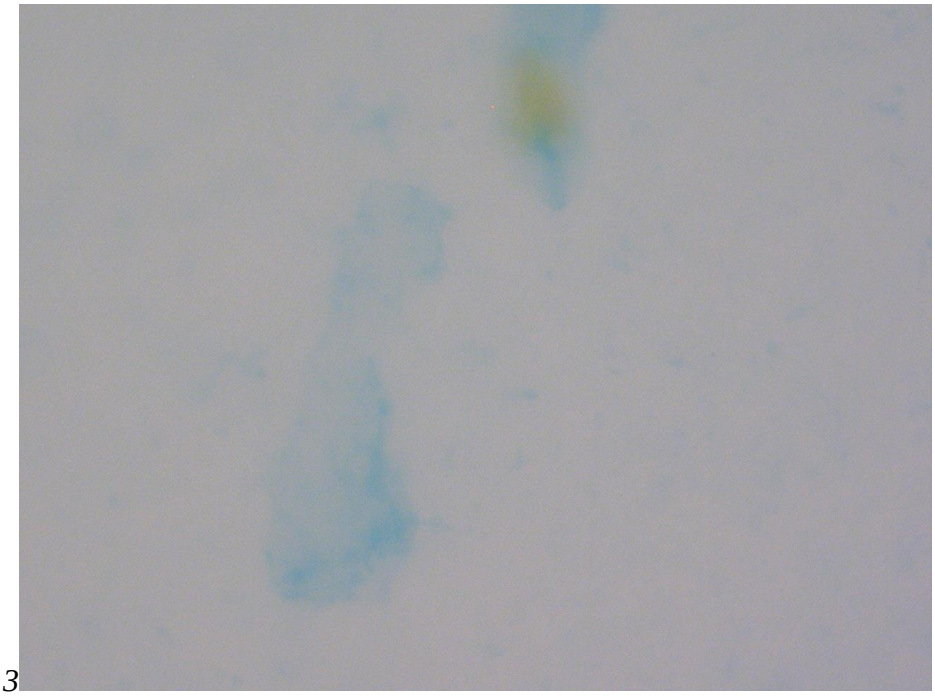


Figure13

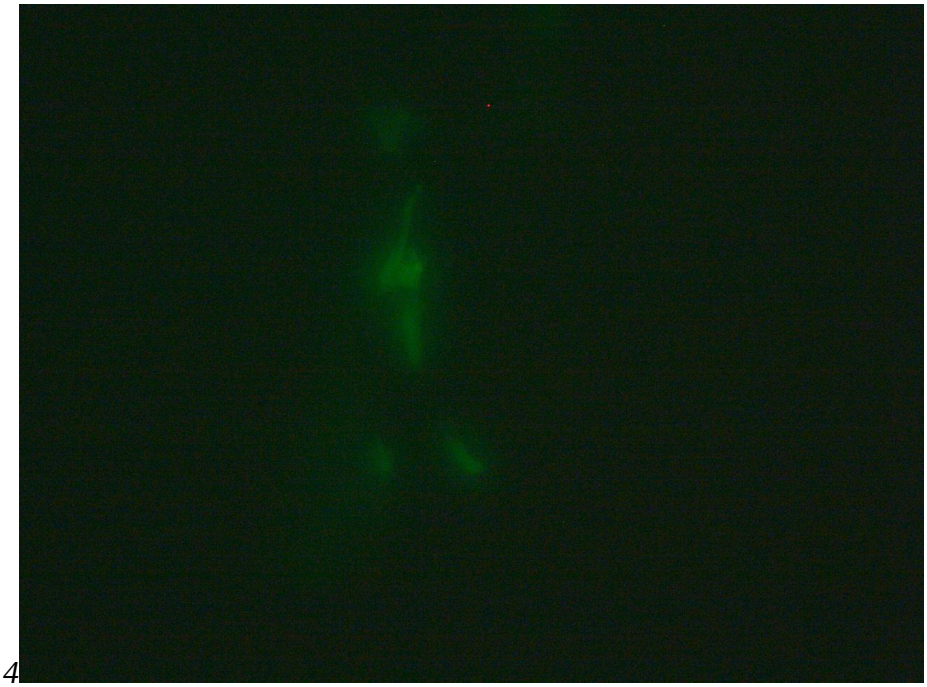


Figure 14 *Odontella aurita* Culture: 4/1/2013

Figure 13: Photo taken under a halogen lamp. Sample stained with Alcian Blue and concanavalin A-FITC

Figure 14: Photo taken under fluorescent GFP filter. Same sample showing the presence of glucose or mannose in the polysaccharide exopolymer particle

Figure15

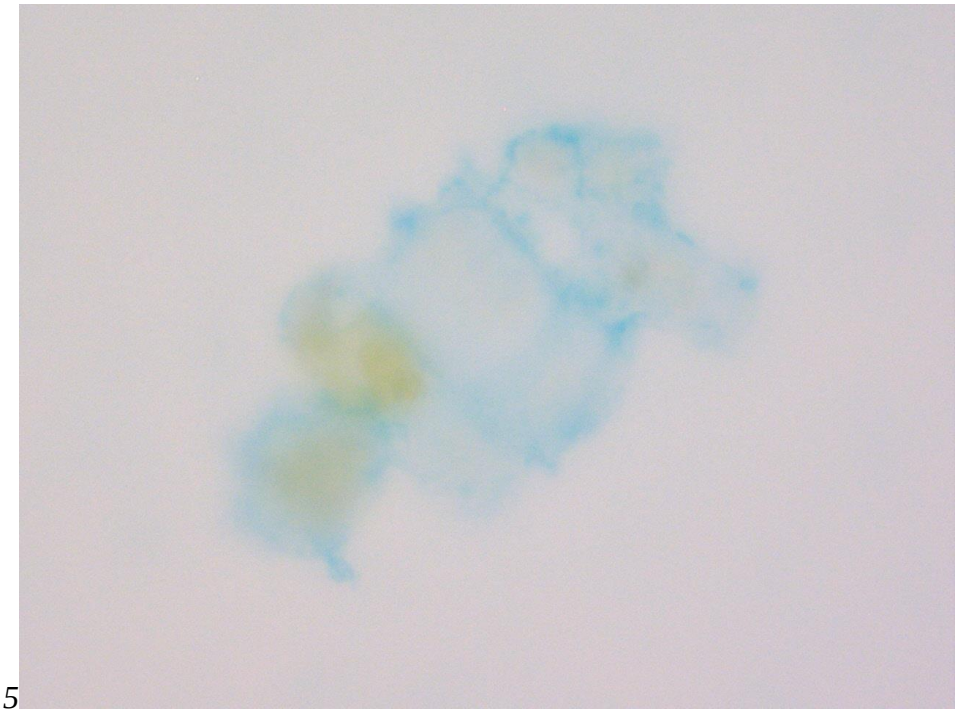
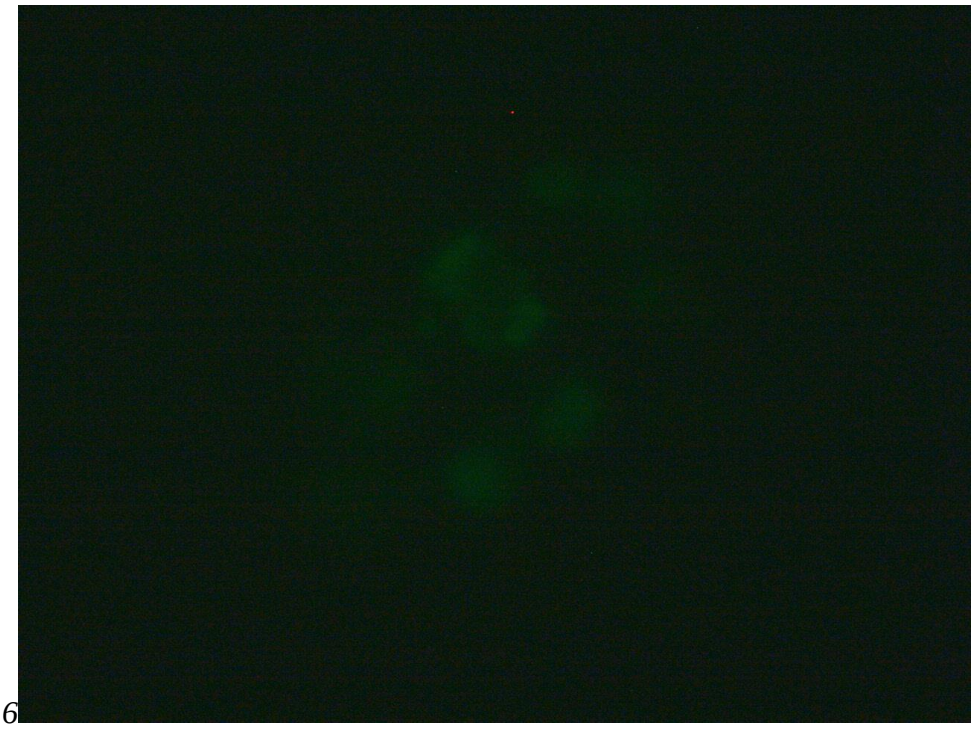


Figure 16



Odontella aurita Culture: 4/1/2013

Figure 15: Photo taken under a halogen lamp. Sample stained with Alcian Blue and concanavalin A-FITC

Figure 16: Photo taken under fluorescent GFP filter. Same sample showing the presence of glucose or mannose in the polysaccharide exopolymer particle.

In the results, there was evidence that there is glucose and mannose in the CSP particles, even though CSP consist of protein exopolymer particles, as shown in Figures 18-21.

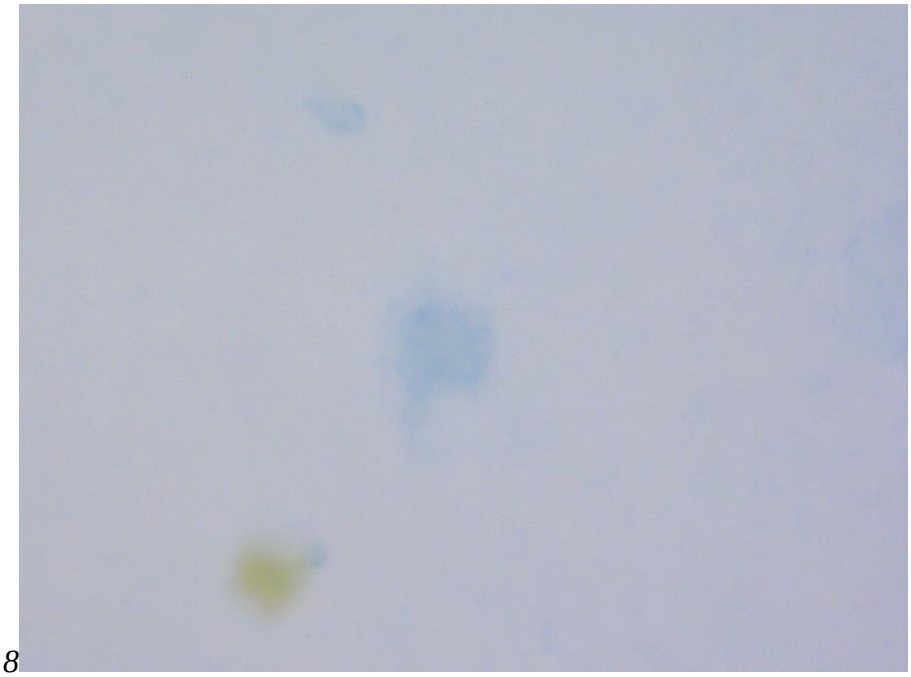


Figure 18

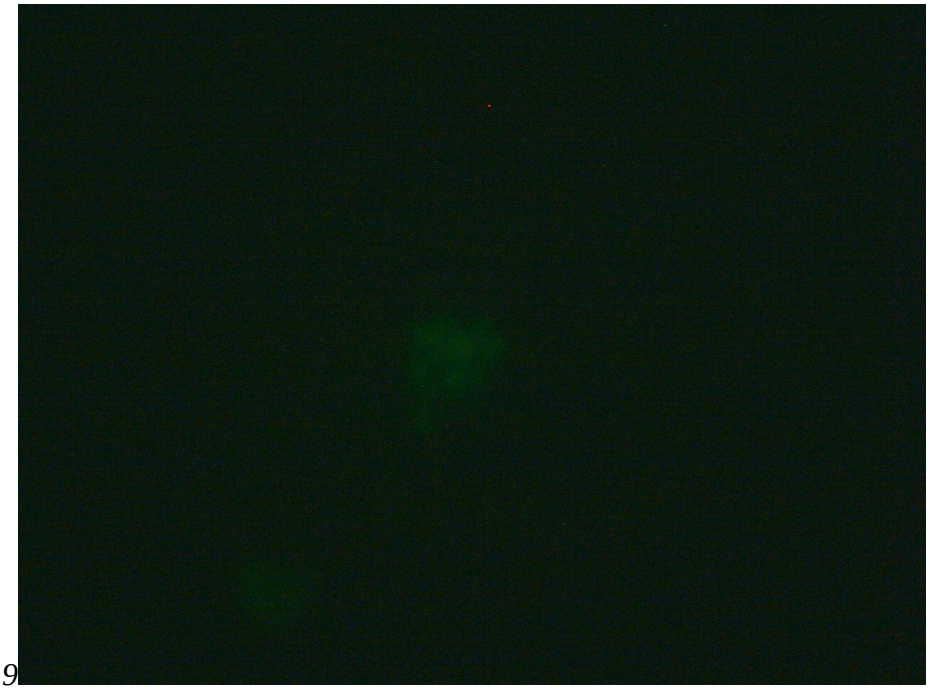


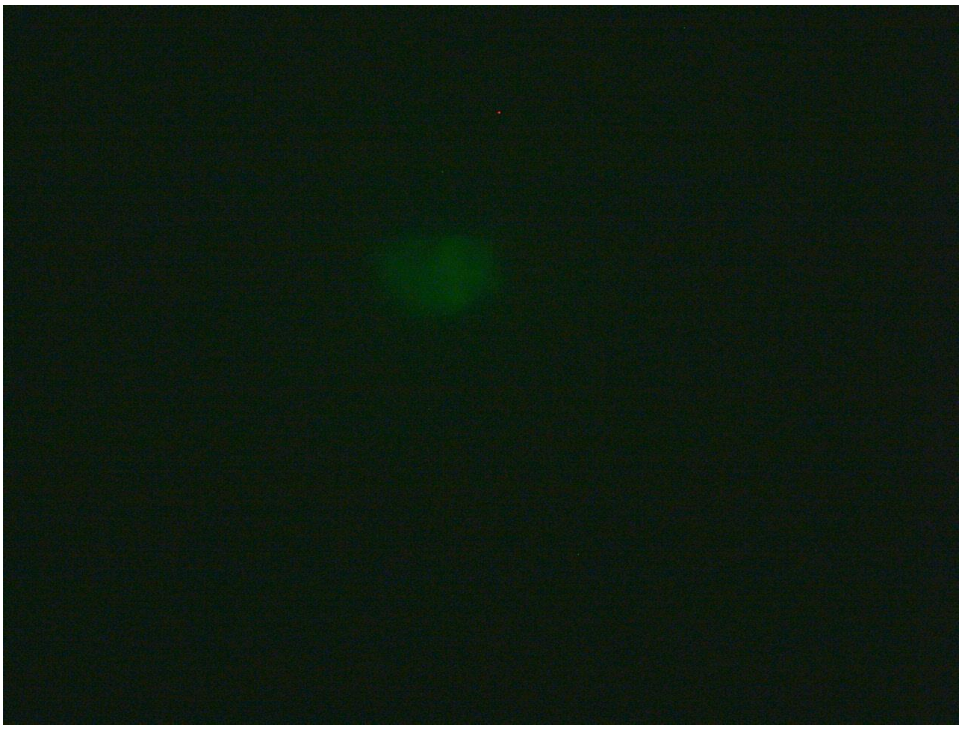
Figure 19 *Odontella aurita* Culture: 4/1/2013

Figure 18: Photo taken under a halogen lamp. Sample stained with Coomassie Brilliant Blue and concanavalin A-FITC

Figure 19: Photo taken under fluorescent GFP filter. Same sample showing the presence of glucose or mannose in the assumed homogenous protein exopolymer particle

Figure 20

Figure 21



Odontella aurita Culture: 4/1/2013

Figure 20: Photo taken under a halogen lamp. Sample stained with Coomassie Brilliant Blue and concanavalin A-FITC.

Figure 21: Photo taken under fluorescent GFP filter. Same sample showing the presence of glucose or mannose in the assumed homogenous protein exopolymer particle.

For every species, there were slides created with just concanavalin A-FITC to determine the amount of glucose and mannose in a sample that does not have the opportunity to react with another stain. In every culture, there was an abundance of glucose and mannose (Figure 22).

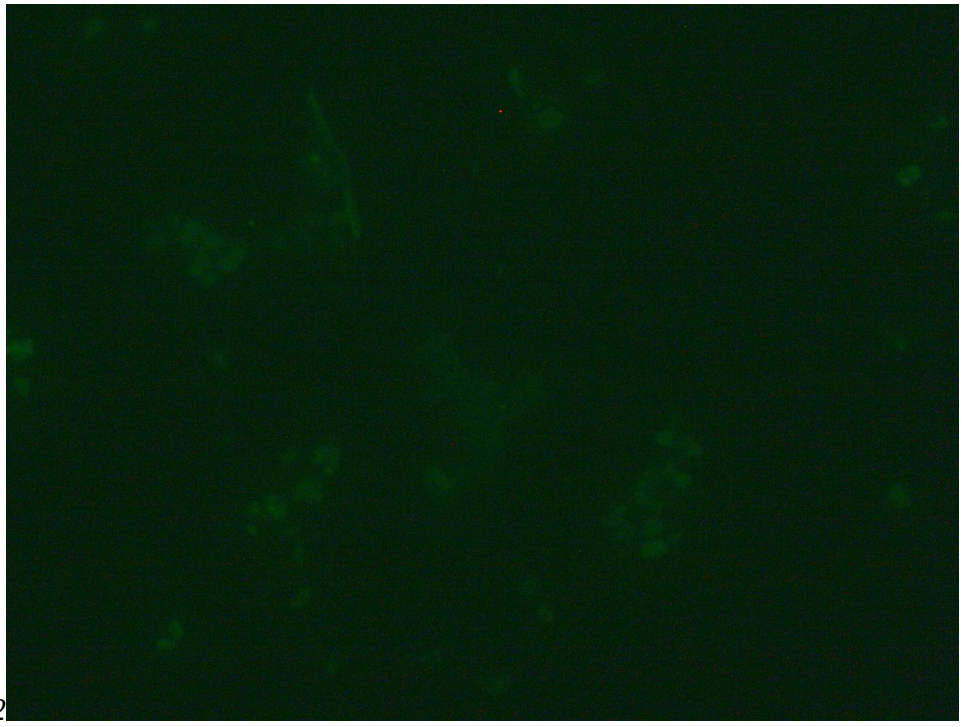


Figure 22 *Odontella aurita* culture: 4/4/2013

Figure 22: Photo taken of the concanavalin A-FITC only slide from the *Odontella aurita* culture. This shows the abundance of glucose and mannose in the sample.

The results from the first experiment disprove the original hypothesis. There was no enough DNA to affect any type of measurement or species analysis. There were minor problems in this experiment concerning precipitates in the medium which fluoresced in the background. This did not interfere with taking the pictures for the qualitative analysis though. This experiment did not prove that exopolymer particles are anything besides the assumed homogenous acid polysaccharides and protein, though the results from the second experiment did. In the test for glucose and mannose derived monosaccharides, it was discovered that contents of TEP and CSP are specific based on species. In the TEP and CSP from the *Odontella aurita* sample, there was lectin, concanavalin A-FITC, fluorescing, expressing that CSP is not just proteins for this species. This proves the hypothesized heterogeneous nature of these particles. Unfortunately, as seen in the concanavalin A-FITC only pictures, it is believed that the acidic nature of the Alcian Blue has reacted with the lectin, causing the TEP amounts of glucose and mannose to be understated. Based on this analysis, the amount in TEP would be short of the true amounts.

Discussion

While the reasons are still unknown for the cause of the excretions, the heterogeneous nature of the sample of CSP from the *Odontella aurita* is important for future studies of the cause and reason for the exopolymer particles. By determining the composition of the particles, it is easier to determine what processes the particles can be used for and given the importance to the food web and biological carbon pump, easier to find how much they influence the global processes. Future studies would include determining further the differences between the species and their production. Also, testing for other monosaccharides in both TEP and CSP would need to be determined to help in understanding why these particles are created. It does not seem logical for

the cell to excrete polysaccharides and proteins that it needs to function. Still, it is not certain why the excretions happen (Gärdes et al 2012). They are extremely important when it comes to the biological carbon pump though because of the recycling of nutrients and the flux of carbon to the deep ocean (Gärdes et al 2012). These particles provide much needed nutrients to the deep ocean and provide little platforms for bacteria aggregations to grown on in the surface waters, though the full impact is not known (Gärdes et al 2012). Uneaten particles can also be buried in the sediments, aiding in the sequestration of carbon in the oceans (Gärdes et al 2012). This is significant since exopolymer particles make up more carbon in the oceans than living organisms (Verdugo et al. 2004). If the exopolymer particles are heterogeneous, then the reason of excretions and their fate in the water column can be affected. This can be affected by any change in the oceans, and therefore the ecosystems, sequestration of carbon, and the nutrient balance will be disrupted, which can affect all life and productivity in the oceans.

CHAPTER IV

CONCLUSIONS

Phytoplankton are very important to the biogeochemical cycles in the ocean. The exopolymer particles that diatoms excrete are an integral part of the oceanic food web and the recycling of nutrients. Based on the results, different species excrete different acid polysaccharides to make up TEP and in some cases; CSP is a heterogeneous structure as well. Extracellular DNA can be found in TEP, but there is no significant amount to affect biomass measurements in the ocean. While the reasons are still unknown for the cause of the excretions, the heterogeneous nature of the sample of CSP from the *Odontella aurita* is important for future studies. This study showed the heterogeneous nature of exopolymer particles produced by marine diatoms. This is influential on the recycling of nutrients and the transfer of energy within the food webs. There is also an effect on the biological carbon pump since the consumers may change based on the composition and the level in the ocean where the particles actually settle or get eaten. Exopolymer particles are extremely important to the environment and the processes within the oceans and this new field will have a huge impact of understanding the futures of the oceans, of our atmosphere, and of our world.

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